Role of myristoylation and N-terminal basic residues in membrane association of the human immunodeficiency virus type 1 Nef protein

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Human immunodeficiency virus type 1 Nef protein is N-terminally myristoylated, a modification reported to be required for the association of Nef with cytoplasmic membranes. As myristate alone is not sufficient to anchor a protein stably into a membrane, it has been suggested that N-terminal basic residues contribute to Nef membrane association via electrostatic interactions with acidic phospholipids. Here, data are presented pertaining to the role of the myristate and basic residues in Nef membrane association, subcellular localization and function. Firstly, by using a biochemical assay for membrane association it was shown that, whereas myristoylation of Nef was not essential, mutation of a cluster of four arginines between residues 17 and 22 reduced membrane association dramatically. Mutation of two lysines at residues 4 and 7 had negligible effect alone, but when combined with the arginine substitutions, abrogated membrane association completely. By using indirect immunofluorescence, it was demonstrated that mutation of either of the two basic clusters altered the subcellular distribution of Nef dramatically. Thirdly, the requirement of the arginine and lysine clusters for Nef-mediated CD4 downmodulation was shown to correlate precisely with membrane association. These data suggest that membrane localization and subcellular targeting of Nef are controlled by a complex interplay of signals at the N terminus of the protein.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) Nef protein is a 205 aa, N-terminally myristoylated phosphoprotein that has been demonstrated to play a critical role in virus replication and pathogenicity. Nef functions can be classified into three categories: (i) effects on protein trafficking, specifically downmodulation of cell-surface molecules such as CD4 and major histocompatibility complex class I; (ii) modulation of signal-transduction pathways such as those emanating from the T-cell receptor; and (iii) enhancement of virus infectivity. Many details of the molecular mechanisms underpinning these effects are known (reviewed by Arora et al., 2002; Fackler & Baur, 2002). A common theme is that they require the association of Nef with cellular membranes, an attribute that Nef shares with many cellular myristoylated proteins. Intriguingly, in both virus-infected cells and cells transfected with Nef expression constructs, a significant proportion of the protein is found in the cytosol (Coates et al., 1997; Kaminchik et al., 1994; Niederman et al., 1993; Yu & Felsted, 1992). It is not known whether these two populations of Nef represent a dynamic equilibrium in which individual Nef molecules shuttle on and off the membrane or whether they are distinct biochemical species with different functions. Subcellular localization studies, together with protein–protein interaction data, support a model whereby Nef accelerates the internalization of cell-surface molecules (e.g. CD4) by interacting with the plasma membrane and then recruiting the cellular endocytic machinery to its target protein. This process targets that protein to a degradative pathway, after which Nef finally recirculates to the plasma membrane. What is not clear is exactly which attributes of Nef govern the first crucial step, i.e. the interaction with cellular membranes. In this context, it has been shown previously that myristoylation alone is not sufficient to anchor a protein stably into a lipid bilayer (Murray et al., 1998) – an additional membrane-targeting signal, such as a cluster of basic amino acids that can participate in electrostatic interactions with acidic phospholipids or a second acylation event such as palmitoylation, is required to stabilize the association. In the case of the basic-cluster mechanism, addition of phosphate groups adjacent to the membrane can negate the electrostatic interaction and result in dissociation of the protein from the membrane. This can be reversed by dephosphorylation and this mechanism is termed a ‘myristoyl switch’ (McLaughlin & Aderem, 1995). We have shown previously that two clusters of basic residues near the N terminus contribute to the virion incorporation of Nef and the ability of Nef to enhance virus infectivity; the latter was determined by using a single-round infectivity assay [multinuclear activation of a
galactosidase indicator (MAGI) and in peripheral blood mononuclear cells (Welker et al., 1998). The requirement for these basic residues was assumed to be due to their role in membrane association; however, at that time, we did not present data to support this hypothesis. Here, we have addressed this question directly by presenting the results of an analysis of the role of these two clusters (Lys4 and -7, and Arg17, -19, -21 and -22) in membrane association, subcellular localization and function of Nef. We demonstrated that, whereas the lysines were dispensable for both association of Nef with cellular membranes and efficient down-modulation of CD4, the arginine cluster was required for both of these processes. However, the fact that this arginine cluster was not adjacent to any serine residues suggests that a phosphorylation-mediated myristoyl switch may not regulate Nef membrane association.

METHODS

Plasmid constructs. The plasmid pCG-NL4-3-IRES-GFP encodes a bicistronic RNA expressing the NL4-3-allele of Nef and enhanced green fluorescent protein (EGFP) separated by the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), under control of the human cytomegalovirus immediate-early enhancer/promoter (Carl et al., 2001). In pCG-NL4-3-STOP-IRES-GFP, two in-frame termination codons have been introduced into the Nef ORF. To introduce mutant Nef alleles, pCG-NL4-3-IRES-GFP was first digested with XbaI and MluI to remove the NL4-3 Nef insert. Nef mutants were generated by PCR from appropriate templates (Welker et al., 1998) with primers incorporating XbaI and MluI sites. Amplimers were digested with XbaI and MluI and ligated into XbaI/MluI-digested pCG-NL4-3-IRES-GFP. To generate plasmids that expressed only Nef and not GFP, pCG-NL4-3-IRES-GFP derivatives were digested with BamHI, gel-purified and religated, thus removing the IRES–GFP segments. Clones of murine Lck (wild-type and the non-myristoylated Gly2-Ala mutant) were generated by PCR removing the IRES–GFP segments. Clones of murine Lck (wild-type for these basic residues was assumed to be due to their role in membrane association; however, at that time, we did not present data to support this hypothesis. Here, we have addressed this question directly by presenting the results of an analysis of the role of these two clusters (Lys4 and -7, and Arg17, -19, -21 and -22) in membrane association, subcellular localization and function of Nef. We demonstrated that, whereas the lysines were dispensable for both association of Nef with cellular membranes and efficient down-modulation of CD4, the arginine cluster was required for both of these processes. However, the fact that this arginine cluster was not adjacent to any serine residues suggests that a phosphorylation-mediated myristoyl switch may not regulate Nef membrane association.

METHODS

Western blotting. Gradient fractions were separated by SDS-PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore) prior to blotting with the following antibodies: sheep polyclonal sera to Nef (Harris & Neil, 1994) or CD4 (obtained from the Centralised Facility for AIDS Research, NIBSC, UK), rabbit polyclonal sera to GFP (BD Biosciences), Lck (Santa Cruz Biotechnology) or ICAM-1 (Tuthill et al., 2003), or a mouse mAb to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam).

Indirect and direct fluorescence. Transfected cells on glass coverslips were washed three times in PBS, fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and washed twice more with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min and subsequently washed twice with PBS. Nef was detected by using an rat anti-Nef mAb (Ab3108, obtained from the Centralised Facility for AIDS Reagents, NIBSC, UK), diluted 1:50 in PBS/10% FCS, for 1 h at room temperature in a dark humidified container. This was followed by three washes in PBS and secondary detection using either Alexa Fluor 594-conjugated donkey anti-rat (Invitrogen) or fluorescent isothiocyanate (FITC)-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories) at a 1:200 dilution in PBS/10% FCS for 1 h at room temperature in a dark humidified container. Hoechst 33258 (Invitrogen) was used at a 1:10 000 dilution to detect nuclei. After staining, cells were washed three times in PBS, once in distilled water and mounted on CitiFluor (Agar Scientific). Digital deconvolution and image analysis were then performed on three-dimensional datasets using ten iterations of a constrained iterative deconvolution algorithm with SoftWorks deconvolution software (Applied Precision).

FACS analysis. HeLa cells expressing full-length CD4 (HeLa-CD4) were transfected with pCG-NL4-3-IRES-GFP or mutant derivatives (Carl et al., 2001). As a negative control, HeLa cells were transfected with pEGFP-N1 (Clontech). Cells were harvested at 48 h post-transfection, washed once with PBS, once with PBS/1% FCS and then incubated with a 1:40 dilution of a phycoerythrin–Cy5-conjugated monoclonal anti-CD4 (CALTAG Laboratories) in PBS/1% FCS. After a 30 min incubation at 4°C, cells were washed three times with PBS/1% FCS and analysed on a Becton Dickinson FACSCalibur with CellQuest software. The levels of cell-surface CD4 were assayed on the GFP-negative and -positive cell populations.

Cell culture. Parental HeLa cells or HeLa cells line expressing either wild-type human CD4 or a truncation mutant lacking the majority of the cytoplasmic domain (CD4STOP399) (Pitcher et al., 1999) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone) supplemented with 10% FCS, 100 IU penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. All transfections were carried out using 2-5 μl Lipofectamine (μg DNA) (Invitrogen). Cells for fluorescence analysis were seeded on to glass coverslips in 12-well plates at a concentration of 8 × 10⁴ cells per coverslip and transfected with 2 μg DNA. Cells to be transfected for fluorescence-activated cell-sorting (FACS) analysis or cellular fractionation were seeded into six-well plates at a concentration of 2 × 10⁵ cells per well and transfected with 2 μg DNA. Three wells of a six-well plate were pooled for each fractionation experiment. For myristic acid labelling, 200 μCi (7-4 MBq) [¹⁴C]myristic acid (vacuum-dried and resuspended in DMSO) per well of a six-well plate was added at 16 h post-transfection. Cells were incubated overnight, washed twice with PBS, trypsinized, lysed in GLB (Bentham et al., 2003) and analysed by SDS-PAGE.

Cell fractionation. Cell membranes were separated essentially as described previously (Spearman et al., 1994). Briefly, at 48 h post-transfection, cells were washed twice with ice-cold PBS and harvested into PBS/10 mM EDTA at 4°C for 5–10 min. Cells were gently pelleted (400 g), resuspended in ice-cold PBS and pelleted again. Cells were suspended in hypotonic buffer [10 mM Tris/HCl (pH 7.4), 1 mM EDTA] supplemented with protease inhibitors (Boehringer Mannheim) and allowed to swell in this buffer for 15–20 min before being disrupted by passing through a 21G needle. Lysis was confirmed by phase-contrast microscopy. The suspension was adjusted to 150 mM NaCl and centrifuged (1000 g, 10 min at 4°C) to pellet nuclei and unbroken cells. Where indicated in the text, SDS was added to the post-nuclear supernatant to a final concentration of 0.1%. The post-nuclear supernatant was adjusted to 80% (w/v) sucrose and layered underneath 65 and 10% sucrose layers prior to ultracentrifugation (151 000 × g for 4 h). Under these conditions, membrane vesicles float to the 65/10% interface and thus membrane-associated proteins are detected at the top of the gradient. These gradients were fractionated and 3 vols methanol was added to each fraction to precipitate the proteins (1 h, −18°C). Proteins were pelleted (20 800 g, 30 min at 4°C) and resuspended directly in SDS-PAGE loading buffer.
RESULTS

A critical role for a cluster of arginine residues in membrane targeting of Nef

We (Welker et al., 1998) and others (Kaminchik et al., 1994; Niederman et al., 1993; Yu & Felsted, 1992) have shown previously by subcellular fractionation and differential centrifugation that wild-type Nef is distributed in both the soluble and membrane-bound fractions of the cytoplasm. Furthermore, we demonstrated that both populations were myristoylated to an equal extent, suggesting that the cytosolic localization could not be explained by lack of myristoylation (Coates et al., 1997). In order to define in more detail the requirements for Nef membrane association, we established a membrane flotation assay that we reasoned would provide a less harsh physical separation of the membrane and cytosolic fractions, as it would not involve high-speed pelleting of membrane fractions. Accordingly, HeLa cells constitutively expressing a CD4 mutant lacking the cytoplasmic tail (CD4stop399) (Pitcher et al., 1999) were transfected transiently with a bicistronic vector expressing both Nef (NL4-3 isolate) and EGFP, the latter under the translational control of the EMCV IRES (Carl et al., 2001). Cells were lysed in hypotonic buffer and lysates were separated by ultracentrifugation. Under these conditions, membrane vesicles and membrane-associated proteins accumulated at the top of the gradient. Fig. 1(a) demonstrates that, although most Nef protein remained at the bottom of the gradient (i.e. cytosolic), a significant proportion (mean of 24%; Table 1) migrated to the 10 % sucrose fraction, demonstrating that it was associated with cytoplasmic membranes. Our data also confirmed that the level of Nef expression had no substantive effect on the amount of Nef associated with membrane [compare Fig. 1a and Fig. 2b(i)]. After treatment of the post-nuclear supernatant with 0-1 % SDS, all of the Nef remained at the bottom of the gradient (Fig. 1b), confirming that the protein in the 10 % sucrose fraction was indeed lipid-associated. To validate the experimental protocol further, gradients were analysed for the presence of GFP or CD4stop399. As expected, all of the GFP remained at the bottom of the gradient (Fig. 1c), whereas CD4stop399 was almost entirely membrane-associated and migrated to the 10 % sucrose fraction (Fig. 1d; Table 1). These data were consistent with the facts that GFP lacks any membrane-association signals and that CD4 is an integral membrane glycoprotein and is, therefore, almost entirely membrane-associated. The CD4stop399 mutant was chosen for this analysis as the lack of a cytoplasmic tail means that it cannot undergo endocytosis and is therefore trapped at the cell surface. Additionally, as we have demonstrated previously, it is unable to interact with Nef (Bentham et al., 2003; Harris & Neil, 1994). As CD4stop399 and GFP were exogenously expressed proteins, we also validated the assay by analysing the gradients for the presence of two endogenous proteins – intercellular adhesion molecule-1 (ICAM-1) and GAPDH. As expected, all of the ICAM-1 was membrane-associated (Fig. 1e), whereas all of the GAPDH was cytosolic (Fig. 1f).

Table 1. Protein distribution between cytosol and membrane fractions

Densitometry was applied to the Western blots shown in Figs 1 and 2 by using Advanced Image Data Analyser version 2.0 software (Raytek Scientific). The total signal for fractions 1–4 and 8–9 was taken as 100 %. The numbers shown represent the percentage of signal in fractions 1–4 (cytosolic) and in fractions 8–9 (membrane-associated).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cytosolic fraction (%)</th>
<th>Membrane-associated fraction (%)</th>
<th>SD (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NefWT</td>
<td>75.7</td>
<td>24.3</td>
<td>1.46</td>
</tr>
<tr>
<td>NefG2A</td>
<td>86</td>
<td>14</td>
<td>1.97</td>
</tr>
<tr>
<td>NefR</td>
<td>77.5</td>
<td>22.5</td>
<td>1.89</td>
</tr>
<tr>
<td>NefR</td>
<td>93.5</td>
<td>6.5</td>
<td>1.12</td>
</tr>
<tr>
<td>NefKR</td>
<td>97.5</td>
<td>2.5</td>
<td>0.95</td>
</tr>
<tr>
<td>NefG2A-K</td>
<td>92.2</td>
<td>7.8</td>
<td>3.53</td>
</tr>
<tr>
<td>NefG2A-R</td>
<td>99.1</td>
<td>0.9</td>
<td>0.36</td>
</tr>
<tr>
<td>NefWT + SDS</td>
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<td>0.2</td>
<td></td>
</tr>
<tr>
<td>LckWT</td>
<td>10.2</td>
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<td></td>
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<tr>
<td>LckG2A</td>
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<td>0.5</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>17.8</td>
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<td></td>
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<td>GFP</td>
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</tr>
<tr>
<td>ICAM-1</td>
<td>0.2</td>
<td>99.8</td>
<td></td>
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<tr>
<td>GAPDH</td>
<td>99.5</td>
<td>0.5</td>
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Fig. 1. Wild-type Nef partitions between the cytosol and cytoplasmic membranes. Lysates from HeLa-CD4stop399 cells transiently transfected with bicistronic Nef-IRES-GFP vectors were fractionated on discontinuous sucrose gradients. Fractions were concentrated by methanol precipitation, separated by SDS-PAGE and analysed by Western blotting for Nef (a, b), GFP (c), CD4 (d), ICAM-1 (e) or GAPDH (f). Lane I represents 10 % of the unfractionated input lysate.
concluded from these data that a subset of the wild-type Nef protein is membrane-associated in vivo.

In order to determine the relative contributions of the myristate moiety and the two clusters of basic residues near the N terminus to membrane association of Nef, we generated a panel of mutants. First, we generated a non-myristoylated derivative in which the myristoyl-acceptor glycine (Gly2) was mutated to alanine (termed NefG2A). Secondly, the two clusters of basic residues were mutated to valine or alanine: mutant NefK contained substitutions of Lys4 to Val and Lys7 to Ala, whereas the NefR mutant contained substitutions of Arg17, -19, -21 and -22 to Ala (for amino acid sequences, see Fig. 2a). These two mutants were made in the context of both wild-type myristoylated Nef and in the background of the Gly2Ala, non-myristoylated derivative (NefG2A-K and NefG2A-R). Lastly, we combined both the lysine and arginine mutations (NefKR). Fig. 2 demonstrates that, somewhat surprisingly, approximately 14 % of NefG2A was membrane-associated (Table 1). Whereas NefG2A-K retained a low level of membrane binding [Fig. 2b(vii)], NefG2A-R exhibited even lower binding than NefKR [Fig. 2b(viii)] (Table 1). These data are consistent with a critical role for the arginine cluster in stabilizing Nef membrane association and a lesser role for the lysines. As the result with the NefG2A mutant was somewhat unexpected, we performed a control experiment to confirm the role of myristoylation in membrane association of a known cellular myristoyl protein, the Src-family tyrosine kinase, Lck, under the same experimental conditions. Lck is not normally expressed in HeLa cells and we reasoned that it was therefore an appropriate control for Nef. HeLa cells were transfected with plasmids expressing either wild-type murine Lck or a derivative in which the myristoyl-acceptor glycine (Gly2) was mutated to alanine (LckG2A). The results showed that essentially all of the wild-type Lck partitioned in the membrane fraction [Fig. 2b(viii)], whereas the Gly2Ala mutation completely abolished membrane association of Lck [Fig. 2b(ix)] (Table 1). These data validated the experimental protocol and confirmed that the membrane association of the NefG2A mutant was a genuine and specific property of Nef and not an artefact of the experimental system. Interpretation of this data would potentially be complicated if the lysine or arginine mutations affected the ability of Nef to undergo cotranslational myristoylation. This was particularly true for

![Fig. 2. An N-terminal arginine cluster is critical for membrane targeting of Nef. (a) Schematic of the Nef mutants used in this study. Residues highlighted in bold were mutated. (b) Lysates from HeLa-CD4stop399 cells transiently transfected with bicistronic Nef–IRES–GFP vectors (i–vii), pSG5.Lck (viii) or pSG5.Lck(G2A) (ix) were fractionated on discontinuous sucrose gradients. Fractions were concentrated by methanol precipitation, separated by SDS-PAGE and analysed by Western blotting for Nef (i–vii) or Lck (viii, ix). Lane I represents 10 % of the unfractionated input lysate. (c) Transiently transfected HeLa cells were labelled with [3H]myristate, lysed and separated by SDS-PAGE followed by autoradiography (top panel) or Western blotting (lower panel) for Nef. Lane M represents mock-transfected cells.](image-url)
the lysine mutant, as substrate recognition by N-myristoyl transferase requires the N-terminal 6 aa of a target protein. To test whether proteins of the expressed Nef mutant panel were indeed myristoylated, transfected cells were labelled with \[^{3}H\]myristic acid. Lysates were separated by SDS-PAGE and analysed by autoradiography or Western blotting to confirm levels of Nef expression. The results shown in Fig. 2(c) confirmed that, with the expected exception of NefG2A, all of the Nef proteins were myristoylated efficiently. Consistent with this, neither NefG2A-K nor NefG2A-R was myristoylated (data not shown). We concluded from these data that, contrary to previous observations, myristoylation of Nef is not required absolutely for membrane targeting. Although both the myristate and the two lysines contributed to membrane binding, the major determinant appeared to be the cluster of arginine residues.

**Both N-terminal basic clusters influence the subcellular distribution of Nef**

Given that the two basic clusters had different effects on Nef membrane association as determined by a biochemical assay, we predicted that they might also result in distinct alterations to the subcellular localization of the protein. Accordingly, we analysed the distribution of the panel of Nef mutants by indirect immunofluorescence and epifluorescent microscopy. As shown in Fig. 3(a), wild-type Nef was distributed at the periphery of the cell (plasma membrane), as well as in a punctate fashion throughout the cytoplasm with a concentration adjacent to the nucleus. This was consistent with previous data showing Nef at the plasma membrane, endosomes and endoplasmic reticulum (ER)/Golgi. In contrast, cells expressing NefG2A showed a much more uniform distribution throughout the cytoplasm, again consistent with previous observations. NefK exhibited a similar diffuse distribution with a perinuclear concentration, suggesting that NefK might target distinct cytoplasmic membranes in comparison with wild-type Nef. The distribution of both NefK and NefKR was similar, with a pattern of diffuse cytoplasmic foci reminiscent of the reticular staining pattern of mitochondria (Griffin et al., 2004); this was more marked for the NefKR mutant. Both NefG2A-K and NefG2A-R exhibited a diffuse cytoplasmic staining similar to that observed for NefG2A.

![Fig. 3. The N-terminal basic clusters influence the subcellular distribution of Nef.](http://vir.sgmjournals.org)
Membrane association correlates with the ability to downmodulate CD4

One of the best-characterized functions of Nef is to increase the rate of endocytosis of CD4, resulting in reduced cell-surface levels of this glycoprotein. It has been assumed previously, but never formally tested, that downmodulation of CD4 requires the membrane association of Nef. To test this, we analysed cell-surface levels of CD4 in populations of HeLa cells expressing wild-type human CD4, transfected with bicistronic Nef–IRES–EGFP vectors expressing either wild-type Nef, a mutant containing several in-frame stop codons (NefSTOP) or the six Nef mutants described above. The use of the bicistronic vector allowed levels of CD4 to be compared directly between cells expressing Nef or not, by gating on either the GFP-positive or GFP-negative populations. As we have shown previously (Bentham et al., 2003), in our hands, expression of wild-type Nef resulted in a reproducible two- to fivefold reduction in the surface levels of CD4 (Fig. 4a), whereas the NefSTOP mutant had no effect, with levels of CD4 on the surface of GFP-positive (Fig. 4a, green line) or GFP-negative (Fig. 4a, blue line) cells being indistinguishable. Consistent with the ability of NefG2A to associate with membranes, we observed that, in comparison with wild-type Nef, this mutant was able to effect a modest downmodulation of CD4. This is also in agreement with previous reports that non-myristoylated Nef retains a low level of CD4-downmodulating activity (AiKen et al., 1994; Iafrate et al., 1997). The ability of NefK to downmodulate CD4 was indistinguishable from that of the wild-type protein, again consistent with the ability of NefK to associate with membranes and confirming a previous report that a K4/7A mutation had no effect on CD4 downmodulation (AiKen et al., 1996). In common with NefG2A, NefR retained a low level of CD4-downmodulation activity; however, in contrast, NefKR was completely inactive. The relative importance of the lysine and arginine clusters was confirmed with the NefG2A-K and NefG2A-R mutants – whereas both were dramatically impaired in their ability to downmodulate CD4, NefG2A-R was less active than NefG2A-K. These data were confirmed by a separate experiment in which the levels of Nef expression were titrated by increasing the amount of plasmid transfected (Fig. 4b, c). Again the phenotype of NefK was wild-type, NefG2A and NefR showed reduced CD4 downmodulation and NefKR was completely unable to downmodulate CD4, even at high expression levels. The phenotypes of the NefG2A-K and NefG2A-R mutants were also consistent with the data in Fig. 4(a). Although others (AiKen et al., 1994; Iafrate et al., 1997) have reported that overexpression of NefG2A can rescue CD4 downmodulation, our data suggest that, at least in HeLa-CD4 cells, this is not the case, as the NefG2A defect relative to wild-type Nef was equally apparent at the three DNA concentrations tested. These data are consistent with the hypothesis that membrane association of Nef is required absolutely for CD4 downmodulation. However, the data suggest that myristoylation may play a more significant role in CD4 downmodulation compared with membrane association, given that the Gly2Ala-containing mutants generally exhibited a greater defect in CD4 downmodulation than the lysine or arginine mutants.

DISCUSSION

The major conclusion from this work is that myristoylation of Nef is not required for the stable association of Nef with lipid bilayers in vivo. Although at first glance this observation seems at odds with the accepted dogma that myristoylation of Nef is absolutely necessary for membrane association, an explanation for this discrepancy may lie in the experimental procedures. Previous studies by our group (Coates et al., 1997; Welker et al., 1998) and others (Kaminchik et al., 1994; Niederman et al., 1993; Yu & Felsted, 1992) utilized a relatively harsh procedure whereby Dounce homogenates were separated into cytosolic and membrane fractions by high-speed centrifugation. It is possible that, for proteins peripherally associated with membranes (e.g. non-myristoylated Nef), this procedure may not allow the preservation of such interactions. In contrast, the relatively gentle technique of membrane flotation is more likely to retain the integrity of both membrane preparations and weak protein–lipid interactions. To validate the assay procedure, we confirmed that a Gly2Ala mutation in the Src-family tyrosine kinase, Lck, completely abolished membrane association [Fig. 2b(viii) and (ix); Table 1]. This contrasted with the situation for Nef, in which the corresponding mutation reduced membrane association by 50 % (Table 1), suggesting that myristoylation of Nef plays a lesser role in membrane association in comparison with cellular myristoyl proteins.

Do non-myristoylated and myristoylated Nef associate with the same membrane compartment? Evidence from studies on other myristoyl proteins suggests that this might not be the case; for example, a non-myristoylated mutant of the yeast Gpa1p protein redirects it from the plasma membrane to intracellular membranes (Song et al., 1996). As the flotation assays used here did not discriminate between plasma or internal cytoplasmic membranes, it is feasible that the
NefG2A mutant undergoes a similar redistribution; indeed, the diffuse distribution of NefG2A (Fig. 3a) supports this hypothesis. However, the observation that, in some cells, NefG2A is also concentrated at the periphery of the cell and is severely compromised in its ability to downmodulate CD4 suggests an alternative explanation – rather than being redirected to intracellular membranes, NefG2A might be impaired in its ability to traffic from the plasma membrane to membranes of endocytic vesicles. This would be in agreement with the results of Faure et al. (2004), who showed that, in comparison with wild-type Nef, the NefG2A mutant failed to fractionate with endosomes on a sucrose gradient. However, these authors did not identify the membrane compartment targeted by NefG2A, although the protein was present in a ‘heavy-membrane’ fraction. This presumably contained all other membranes present in the post-nuclear supernatant (including ER, Golgi, plasma membrane and mitochondria), as well as all of the cytosolic proteins. Thus, the membrane compartment targeted by NefG2A remains elusive. It is interesting to note that the distribution of NefG2A-K did not appear to be significantly different from that of NefG2A or NefK, suggesting that the myristate residue and the lysine cluster did not cooperate to mediate specific membrane targeting.

It is generally accepted that clusters of basic residues in myristoyl proteins participate in electrostatic interactions with acidic phospholipids, the latter being enriched in the inner leaflet of the plasma membrane and in the membranes of endosomes (McCabe & Berthiaume, 1999). In this regard, all of the basic-cluster mutants (NefK, NefR and NefKR) showed a distribution that was distinct from that of the

**Fig. 4.** Membrane association correlates with the ability to downmodulate CD4. (a) HeLa cells stably expressing wild-type human CD4 were transiently transfected with the indicated bicistronic Nef–IRES–GFP vectors and labelled with a phycoerythrin–Cy5-tagged antibody to CD4 (CALTAG Laboratories). Blue lines show CD4 labelling of GFP-negative cells and green lines show labelling of GFP-positive cells. Black lines are the parental HeLa cell line taken through the same staining procedure. (b) Cells were transfected with increasing amounts of Nef–IRES–GFP expression plasmids. The level of surface CD4 for the 0 μg transfection point of pCG-NL4-3STOP-IRES-GFP was taken as 100%. One representative experiment is shown. (c) The geographical mean fluorescence, from three separate experiments, was determined for CD4 expression at the 2 μg transfection points and presented as a histogram.
wild-type in that they lacked the higher concentrations at the plasma membrane and perinuclear region (ER/Golgi). In the case of the NefKR mutant, this aberrant targeting resulted in accumulation in, or in association with, mitochondria, although it is pertinent that the NefG2A-R mutant did not exhibit the punctate staining of NefR, suggesting that myristoylation may play a role in this mitochondrial targeting. Although further work could be carried out to identify the precise localization of these Nef mutants, these studies would be of limited utility in elucidating the role of the two basic clusters in correct targeting of wild-type Nef. Functional analysis of these mutants might be more informative; in this regard, it is interesting to note that, in a previous study (Welker et al., 1998), both the NefK and NefR mutants were defective for Nef-mediated enhancement of infectivity in a single-round MAGI assay system, although when virion incorporation of Nef was assayed by Western blotting of purified virus particles, the NefK defect was more pronounced than NefR. In contrast, the current study showed that only NefR exhibited a defect in CD4 downmodulation. It is possible, therefore, that the lysine and arginine clusters could play roles in targeting Nef to different membrane compartments where it performs different functions, such as CD4 downmodulation and virion incorporation/infectivity enhancement. Lipid rafts or detergent-resistant microdomains are attractive candidates for one of these compartments, especially given the reported role of rafts in HIV assembly and release (Ono & Freed, 2001). Although two groups have indeed shown association of a small proportion of Nef with lipid rafts (Krautkrämer et al., 2004; Wang et al., 2000), we failed to observe such an association (data not shown). Furthermore, it was shown recently that the effects of Nef on both CD4 downmodulation and virus infectivity were independent of rafts (Sol-Foulon et al., 2004). Further work will thus be required to build up a more detailed understanding of the role of these basic clusters in subcellular targeting of Nef.

The critical role for the arginine cluster in membrane association of Nef is consistent with structural data showing that this cluster is within a short α-helix such that three of the arginines (positions 17, 21 and 22) are located on one face of the helix (Geyer et al., 1999). Furthermore, this supports the hypothesis that a phosphorylation-driven myristoyl switch might regulate the subcellular localization of the protein. However, there are no serine or threonine residues proximal to the arginine cluster that could act as phospho-acceptors; the nearest are Ser6, -8 and -9 (see Fig. 2a). In the myristoylated alanine-rich C kinase substrate (MARCKS) protein, which utilizes a well-characterized myristoyl-switch mechanism, the key serine residues phosphorylated by protein kinase C are embedded within the basic region (McLaughlin & Aderem, 1995). It seems unlikely, therefore, that phosphorylation of Nef at Ser6, -8 or -9 could negate the electrostatic interactions between Arg17, -19, -21 and -22 and acidic phospholipids. The lack of requirement for Lys4 and -7 supports this conjecture. The mechanism controlling Nef membrane association thus remains to be elucidated; however, given the reported ability of Nef to oligomerize (Arol et al., 2000), it is intriguing to speculate that an ‘entropic switch’ such as that recently described for the HIV-1 matrix protein (Tang et al., 2004) might also regulate the exposure of the Nef myristate.

Our data clearly demonstrate that the arginine cluster is required for Nef-mediated CD4 downmodulation; indeed, there was a strong correlation between membrane association (Figs 1 and 2b) and CD4 downmodulation (Fig. 4). A simple explanation for this observation is that membrane targeting is required to locate Nef in close proximity to the cytoplasmic tail of CD4, facilitating the direct interaction between Nef and CD4 and thereby promoting downmodulation. Two other studies have used mutagenesis to analyse the role of the arginine cluster in CD4 downmodulation. Iafrate et al. (1997) used a Nef isolate (NA7) derived from an asymptomatic patient that contained the sequence R17ERMRR, somewhat different from the sequence conserved in the majority of the ~1000 Nef alleles in the Los Alamos HIV database (R17ERMRR). Intriguingly, they demonstrated that mutation of this sequence to AAA reduced CD4 downmodulation. In contrast, Aiken et al. (1994) used a more ‘conventional’ (albeit undefined) Nef isolate containing the R17ERMRR sequence and showed that mutation of R17ER→AAA reduced CD4 downmodulation by approximately twofold, whereas an R21A→AA mutant retained wild-type CD4-downmodulation activity. Further confusion surrounds Lys4/7: Iafrate et al. (1997) showed a twofold reduction in CD4 downmodulation when these (together with Arg8 present in NA7 Nef) were mutated to alanine, whereas Aiken et al. (1994) showed that the Lys4/7Ala mutation had no effect. One explanation that would reconcile these conflicting data with our own results involves a consideration of the overall number of positively charged residues at the N terminus of Nef. All three isolates [NA7, the undefined isolate of Aiken et al. (1994) and NL4-3 used in this study] have six basic residues within the first 22 residues of Nef—in the NA7 isolate, the lack of arginines at 21 and 22 is compensated for by a lysine at position 18 and a further arginine at position 8. CD4-downmodulation activity is only maintained if at least three of these basic residues are retained. This might also explain the more dramatic effect of the NefR mutant on membrane association, in comparison with NefK.

In conclusion, our data demonstrate that membrane association and targeting of Nef are complex, functionally relevant processes, involving multiple signals in the N terminus of Nef, and are not, as has been assumed previously, solely due to myristoylation. It may also be the case that the different membrane-association signals of Nef are required to direct Nef to different membrane compartments. Further experiments, such as more detailed mutagenesis and in vitro experiments utilizing purified Nef proteins together with artificial, chemically defined liposomes, will help to unravel these complexities. Such studies are currently under way in our laboratory.
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